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Farelerde Kadmiyum ve Karbon Tetraklorür ile Oluşturulan Oksidatif Hasara Kefirin Etkisinin Belirlenmesi

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Özet: Bu çalışmanın amacı karbon tetraklorid (CCl_4), kadmiyum (Cd), CCI_4-kefir, ve Cd-kefir verilen farelerin eritrositlerindeki glutatyon peroksidaz (GSH-Px) ve katalaz (CAT) aktivitesini ölçmek, redükte glutatyon (GSH) düzeylerini saptamak ve lipid peroksidasyonunun bir indikatörü olan tiyobarbitürik asit ile reaksiyon veren maddelerin (TBARS) düzeylerini belirlemektir. Fareler, her grupta 10 adet hayvan olacak şekilde 5 gruba ayrıldı. Grup I farelerine normal diyet, grup II'ye CCl_4 içeren su, grup III'e $CdCl_2$ içeren su, grup IV'e CCl_4+kefir içeren su ve grup V'e $CdCl_2$+kefir içeren su verildi. Grup II ve grup III'de kontrol grubuna kıyasla plazma TBARS ve GSH düzeyleri önemli oranda ($P<0.001$) daha yüksek bulundu. Eritrositlerdeki GSH-Px aktivitesi kefir verilen gruplarda (grup IV ve V) sadece Cd ve CCI_4 verilen gruplara göre daha düşük bulunurken kefir verilen gruptaki eritrosit CAT aktivitesinde ise artış görüldü. Bu da CCI_4 ve Cd verilen farelerdeki oksidatif stresin azalmasında kefirin etkili olduğunu göstermekte ve kefirin Cd ile CCI_4 toksisitesinden dolayı artan tiyobarbitürik asit substratlarındaki azalmalara önemli bir etkide bulunabileceğini düşündürmektedir.

Anahtar sözcükler: Kefir, Kadmiyum, Karbon Tetraklorid, Antioksidan Enzimler, Serbest Radikaller

Determination of Effect of Kefir on Cadmium and Carbon Tetrachloride-induced Oxidative Damage in Mice

Abstract: The aim of our study was to determine the levels of thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation, to measure the activity of glutathione peroxidase (GSH-Px), catalase (CAT) and to establish the levels of reduced glutathione (GSH) in the red blood cells (RBC) of carbon tetrachloride-treated mice (CCl_4), cadmium (Cd), CCl_4-kefir, Cd-kefir. Mice were divided into 5 groups as 10 animals in each group. The group I was given a normal diet and the group II was given tap water with CCl_4 and the group III was given tap water with $CdCl_2$ and the group IV was given tap water with CCl_4+kefir and the group V was given tap water with $CdCl_2$+kefir. The TBARS and GSH level in the plasma was significantly higher in the group II and III than control group ($P<0.001$). GSH-Px activity in RBC was lower in the kefir groups (group IV and V) than Cd only and CCl_4-only groups only. However, the CAT activity of RBC in kefir administered group was increased. Therefore, the present study demonstrates the effectiveness of kefir in reducing oxidative stress in CCl_4 and Cd-treated mice and suggestes that reductions in increased TBARS due to Cd and CCl_4 toxicity may be an important factor in the action of kefir.

Keywords: Kefir, Cadmium, Carbon Tetrachloride, Antioxidant Enzymes, Free Radicals

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Introduction

Reactive oxygen species generated naturally or introduced into living cells, effect lipid component of polyunsaturated fatty acids (Clarkson and Thompson, 2002; Kasai et al., 1986) and species play an important role in the etiology of many diseases (Nishida et al., 1986). Reactive oxygens play an important role in the mechanism of cellular damage caused by hepatotoxic agents such as carbon tetrachloride (CCl_4) and cadmium (Cd) (Rikans et al., 1994; Güven and Yılmaz, 2005). The toxicity of CCl_4 is generally understood in relation to the metabolic activation of this compound to a trichloromethyl radical, which consequently facilitates lipid peroxidation (Dudely and Klaassen, 1984). The elevation of plasma and tissues lipid peroxidation (LPO) levels is an indicator of membrane disruption in various tissue and organ cells. It is positively correlated with the gravity of the disease. Cadmium a redox-inactive nonessential metal known to be most toxic environmental pollutant is present in soil, air, water and cigarette smoke, and even in food. Cd is listed as one of 126 priority environmental pollutants (Stohs et al., 2001). Cd and CCl_4 accumulates and proves to be a multitarget toxicant causing damage to many organs, such as liver, kidney, lung, brain, bone, placenta, etc. (Lyn Patrick, 2003; Nakagawa, 1993; Ersan et al., 2008.). The results of *in vitro* and *in vivo* studies demonstrated that Cd induces on oxidative stress those results in oxidative deterioration of biological macromolecules (Muler, 1986).

Kefir grain starters consist of a complex community of various bacterial and yeast species. Homofermentative lactobacilli are the main components of the bacterial micro flora (Angulo et al., 1993). Several research studies brought to light some beneficial effects of kefir. Kefir has been used for the treatment of gastrointestinal and metabolic disorders, atherosclerosis, allergic diseases, and tuberculosis (Mccay et al., 1984; Güven and Güven, 2005).

In this study, we investigated the effect of supplementation of kefir on the antioxidant enzyme activities and lipid peroxide levels in mice blood.

Material and Methods

1. Animals and Collection of Samples

Three week old clinically healthy female Swiss Albino mice (n: 50) weighing 25-30 g were used in this study. They were housed 5 mice to a cage, in stainless-steel wire-mesh cages in a temperature-controlled room at 24±2°C with 55% relative humidity and a 12 h light-dark cycle. All animals were given a basic diet.

The animals were allowed to acclimatize for 15 days and randomly assorted into following groups:

Group I (n: 10): Animals were put on a normal diet and with 2 ml/kg distilled water through oral gavages, daily for 12 weeks; this group of animals served as control. Group II was given tap water with 1.5 ml/kgbw CCl_4 for 12 weeks. Also, Group III was given tap water with 3 g $CdCl_2$/L for 12 weeks. Group IV was given tap water with 1.5 ml/kg CCl_4+30 ml kefir and Group V was given tap water with 3 g $CdCl_2$/LU+30 ml kefir daily for 12 weeks.

After 12 weeks of exposure, groups were sacrificed via exsanguination by cardiac puncture into heparinized vacationer syringes (LDM; Paris Franc). Anticaogulanted blood was separated into plasma and red blood cells (RBC) by centrifugation (at 1500x g for 15 minuts). The RBC fractions were washed three times in cold insotonic saline. RBC samples were hemolyzed with a nine-fold volume of redistilled water. Plasma and hemolyzed RBC were stored at -30^{o}C.

2. Kefir preparation

Raw milk was obtained from Kafkas University, Veterinary Faculty Medicine Dairy Farm (Kars, Turkey), and heated to 90^{o}C for 10 min in a water bath, then couled to inoculation temperature (25^{o}C) and 5% kefir grains added. The inoculated milk was incubated at 22^{o}C for

20 h. At the end of the incubation, the grains were separated from the fermented kefir milk by filtration through a plastic sieve and washed prior to the next culture passage (subculture). Kefir drink maintained at 4°C for 24 h and then used for microbiological and chemical analysis before used for feeding the group 3 animals. Only a three days old kefir samples were used for experiments (Marshall and Cole, 1985).

3. Samples processing

25 ml of kefir drink was mixed within 225 ml peptone water (Oxoid Ltd., Hampshire, UK). Tenfold serial dilution from this homogenate was made in the same solution and 0.1 ml from these dilutions tubes spread plated onto separate duplicate plates.

APC were determined using plate count agar (Oxoid, CM 463) incubated at 35°C for 48 h. then all colonies were counted (Harrigan and Mccance, 1976). Samples were plated on MRS agar (Oxoid, CM 361) for lactobacilli and the plates were incubated at 30°C under anaerobic conditions for anaerobiosis, using a 3.5 L anaerobic jar and anaerobic M17 agar (Oxoid, CM 785) was used for the lactic streptococci. The plates were incubated at 30°C. For the enumeration of enterococci Stints and Bratty agar (Oxoid, CM 377) were used. The plates were incubated at 35°C for 48 h. after which typical colonies (pink or dark red, with a narrow whitish border) were counted according to the manufacturer instructions (Oxoid, 1995). Coli forms were determined using violet red bile agar (Oxoid, CM 207) incubated at 37°C for 24 h. The round from red to pink colonies, 0.5-2 mm in diameter and surrounded with a red to pink halo were counted. Selective enumeration of yeasts was achieved using potato dextrose agar (Oxoid, CM 139). The plates were incubated at 22°C for 5 days, and all colonies were enumerated (Marshall and Cole, 1985).

4. Chemical analysis of kefir drink

The water, dry matter, total crude, fat, total lactose and mineral contents of kefir drink were determined using standards (Oxoid, 1995; Oysun, 1991).

5. Analytical Procedures

Lipid hydroperoxide contents were measured with TBARS in plasma by the method of Placer et al. (Placer et al., 1966) and were expressed in terms of the malondialdhyde (MDA) content, which served as a Standard of 1, 1, 3, 3-tetraethoxy propane (Sigma). The values of MDA- reactive material were expressed in terms of TBARS (nmol/mL) plasma. GSH-Px (EC 1.11.1.9) activities of the RBC, samples were measured spectrophotometrically at 37°C and 412 nm according to Lawrence and Burk (Lawrence et al., 1987). The reduced GSH levels of hemolyzed RBC were measured spectrophotometrically using Ellman's reagent (Sedlak and Lindsay, 1968). The hemoglobin concentration in lyses erythrocytes was determined by cyanmethemoglobin method (Canan, 1958). Catalase (CAT EC 1.11.1.6) was expressed as k/gHb (1µmol Hydrogen peroxide loss/min at 25°C). The decomposition of H_2O_2 was followed directly by decreased absorbance at 240 nm, resulting from enzymatic decomposition of H_2O_2. The difference in absorbance (ΔA_{240}) unit time was a measured of catalase activity (Aebi, 1984).

6. Statistical Analysis

The collected values (mean± SE) were statistically analyzed with student's t-test (IFFC, 1987) and evaluated by SPSS 6.0 (SPSS Inc.1993) packed program. P value < 0.01 and 0.05 were considered significant.

Result

At the end of the microbiological analysis of kefir, the average of total mesospheric aerobic colony counts, lactic acid bacteria, lactic streptococcus, enterococcus, total coli form and yeasts were found to be $1.04x10^9$, $9.87x10^8$, $4.38x10^8$, $7.80x10^4$, $1.26x10^5$ CFU/ml respectively. The chemical composition of kefir components consist of water 86.0-89.0%, dry matter 11.0-14.0, lipid 2.8-3.3, total lactose 1.7-2.9.

CCl_4 and $CdCl_2$ show significant changes on antioxidant system components in the red blood cells and plasma of mice. There were significant changes in GSH, GSH-Px and CAT

levels in RBC of Kefir treated groups (P<0.01). Analysis of plasma for lipid peroxidation products revealed a significant (P<0.001) decrease in TBARS concentrations of group IV and V in comparison to group I and II. GSH levels of group II (3.49±0.9 nmol/L) and group III (3.73±0.22 nmol/L) were found to be significantly higher than in control animals (1.66±0.02). Although, lowering of GSH, GSH-Px and CAT were measured in group IV and V than group II and III animals, a significant difference between group IV and V group was not obtained.

Table 1. Activity of antioxidant enzymes and levels of GSH with peroxidation products (TBARS) in plasma of CCl_4 , Cd, CCl_4-kefir and Cd-kefir treated mices

Tablo 1. CCl_4 , Cd, CCl_4-kefir ve Cd-kefir verilen farelerin plazma peroksidasyon ürünleri (TBARS) ile GSH seviyeleri ve antioksidan enzimlerin aktivitesi

GROUPS (n=50)	Plasma TBARS (nmol/ml)	RBC GSH (nmol/ml)	RBC GSH-Px (U/gHb)	RBC CAT (k/mg Hb)
Control	0.93±0.19	1.66±0.02	32.14±2.04	30.3±6.75
Group II	3.27 ±0.50[a]	3.49±0,9[a]	56.80±9.65[a]	42.2±4.14[a]
Group III	2.44±0.52[a]	3.73±0.22[a]	48.54±7.13[a]	40.9±7.12[a]
Group IV	2.02±0.1[b]	2.83±0.9[b]	31.33±7.18[c]	36.3±3.29[b]
Group V	1.67±0.17[b]	2.1±0.2[b]	31.60±5.9[c]	35.7±3.21[b]

$P<0.05^c$, $P<0.01^b$, $P<0.001^a$

Note: values are expressed as mean ± SD

Discussion

Function of related to kefir to against toxic agents, oxygen, CCl_4 and Cd were extensively studied. Conclusions drown from this study to date the importance antioxidant kefir. However, there is little record about antioxidative characteristic of kefir.

This study also indicates that the kefir-Cd and kefir-CCl_4 complex are less or essentially nontoxic to the LPO. Lipid peroxidation is complex process in that oxidation of polyunsaturated fatty acids of membrane lipid leads to membrane damage and cell death (Manno et al., 1985; Yerlikaya et al., 2008). In this experiment, marked elevation of GSH-Px and CAT activity in erythrocytes, MDA concentration in plasma indicated a significant enhancement of LPO processes in the blood.

Cd depletes glutathione and protein-bound sulfhydryl groups, resulting in enhanced production of reactive oxygen species such as superoxide, hydroxyl radicals, and hydrogen peroxide (Shimizu and Morita, 1992). Cd-induced oxidative damage in erythrocytes cases loss of membrane function by enhancing lipid peroxidation and altering the erythrocyte antioxidant system (Güven et al., 2003).

The relative importance of LPO in Cd and CCl_4-induced hepatotoxic was also confirmed in mice pretreated with kefir. Oral kefir pretreatment has been used in this study and protect against the addition, kefir was determined to increase GSH levels in the blood. Kefir was shown to protect against Cd and CCl_4-induced LPO. Though the precise mechanism of this protective effect is unknown, our data suggest that protection resulted from the combination of two kefir sympathized.

The results indicate that the radical scavenging ability of the intact cells and of kefir of lactic acid bacteria (*L. acidophilus*) contributes to the antioxidetive effect (Manno et al., 1985; Shimizu and Morita, 1992). Our results also indicate that these Lactic acid bacteria may have the potential to inhibit the cytotoxicity of CCl_4, Cd and other oxidants as suggested by several authors (Güven et al., 2003; Lin, 1995; Ahotupa et al., 1986).

Glutathione (as a co substrate for GSH-Px) plays an essential protective role against reactive oxygen species that are generated during the metabolism of many xenobiotics. This protective mechanism results in an increased formation of intracellular GSH-Px (Lin and Change, 2000). Although decreased GSH levels have been report in the liver after CCl_4 administration, a literature review failed to

yield any data concerning the effect of hepatotoxines such as CCl_4 on GSH levels in erythrocytes. Likewise, CCl_4 has been reported to repress GSH levels in the rats after administration (Kosower and Kosower, 1978). Similarly as shown in group II, We also found a decrease in the levels of GSH and increase in LPO due to CCl_4 in the plasma of mice after 12 weeks. Nishida et al (1986) reported an increase in peroxyl radicals in plasma when rats were administered with CCl_4. Similar results were observed in the erythrocytes of rats by Sarker et al (1997), reported that Cd dosing increased erythrocyte LPO for up to 3d after a single dosed of $CdCl_2$ (0.4 mg/kg body wt ip), but after 3d of dosing the LPO decreased towards normal.

The GSH-Px activity of the kefir-deficient group was also significantly increased during the depletion diet period when compared with the group II ($P<0.01$).

In summary, the results reported have demonstrated that exposure of mice to moderated doses of Cd. CCl_4-induced LPO, GSH, GSH-Px and CAT in blood of mice. The significant increase of GSH-Px and CAT activity in erythrocytes varied from 44.84, 13.98% for the CCl_4 + kefir group to 43.69, 12.71% for the Cd + kefir group in comparison to the group II and III.

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